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Immunological study of Globo-H, a breast tumor associated carbohydrate antigen, has been producing promising results. As the demand for the supply of this antigen grows, the efficient production of enough material has become an urgent problem. We have been trying to solve this problem by two approaches: the solid support synthesis of Globo-H, and the development of efficient conjugation methods. For the solid support synthesis, solution phase direct roll-over reactions have been performed to verify the possibility that this method can be used to install the 2-amino-2-deoxy galactose moiety on the polymer support. From the results of these reactions, a tumor associated nonasaccharide antigen KH-1 has been synthesized. To improve the conjugation yield of Globo-H to a carrier protein KLH (keyhole limpet hemocyanin), a model study has been carried out to implement an N-hydroxy succinimide ester conjugation method. At the same time, sufficient amounts of Globo-H have been synthesized by solution phase protocol for further immunocharacterization.

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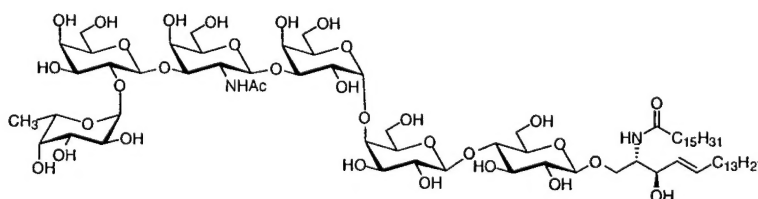
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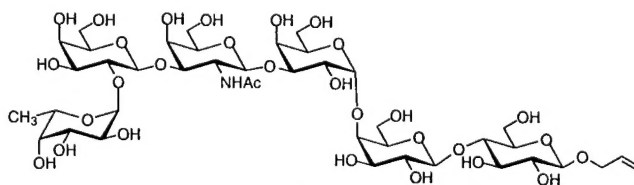
Introduction:

It is common knowledge that carbohydrates play significant roles in intracellular and intercellular recognition.¹ Understanding these roles in the context of cancer immunology and developing cancer vaccines based on the immunological findings has been one of major recent endeavors in the Danishefsky group.

In this regard, the synthesis of breast cancer associated hexasaccharide antigen Globo-H **1** and its derivative allyl glycoside **2** was accomplished² by the glycal methodology,³ and further investigations have been carried out in collaboration with colleagues in the immunology department. Much progress has been made toward the usage of this antigen in cancer immunotherapy. Globo-H antigen was shown to elicit humoral responses in mice,⁴ and a new monoclonal antibody has been raised.⁵ Finally, clinical studies were launched, and the preliminary results from these studies are very promising.⁶ Thus, the supply of this antigen, which is virtually impossible to obtain from natural sources, has become an urgent problem. The results from the studies to solve this problem are described here.



1



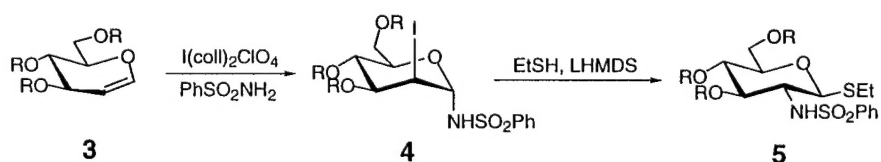
2

I. Solid Phase Synthesis of Globo-H Hexasaccharide

The solid phase synthesis of Globo-H **1** was proposed and actively pursued to obtain sufficient amounts of Globo-H for future studies. Solid support synthesis of carbohydrates in general, however, remains as one of the most challenging problems for synthetic chemists, as no generally applicable method has been developed.⁷ Thus, we decided to continue the solution phase synthesis of Globo-H, so as to improve the steps that may be difficult to apply to the solid phase synthesis.

One such step is the installation of 2-amino-2-deoxy glycoside. Thioglycoside coupling method and direct roll-over method have been used to install the 2-amino-2-deoxy glycoside in the solution phase carbohydrate synthesis (Scheme 1).^{*} Both methods utilize the common intermediate 2-iodo-1-sulfonamide **4**⁸ that can be readily made from glycal **3**. Thioglycoside **5** can be easily made by treatment of **4** with basic ethanethiolate.

Scheme 1



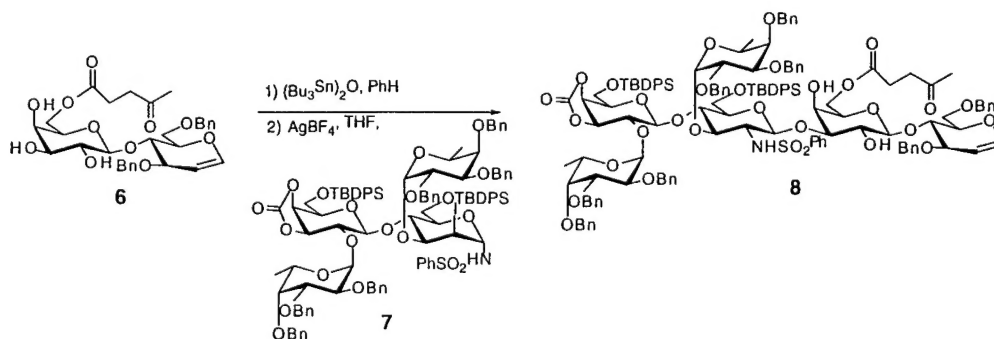
Thioglycosides were proven to be effective donors in various glycosidation reactions. One of the disadvantages of this thio donor is, however, its lack of control in the stereochemical output of the coupling. In many cases, the sulfonamide group, that was hoped to direct the stereochemical output of the coupling, turned out to be inefficient in providing the anticipated anchimeric assistance in the coupling event. So, most thio couplings yield random mixtures of α and β glycosidic linkages. On the other hand, direct roll-over method has been proven to provide

^{*} Acronyms are listed on p 12.

only β linkages due to its stereochemically well defined aziridine (or aziridinium) intermediates. However, this advantage in stereocontrol is accompanied by a lack of reactivity of these donors. Thus, direct roll-over coupling was typically employed with large excess of activated acceptor (around 10 to 20 equivalents) to achieve good yield of coupling product. Chromatographic recovery of excess acceptor was necessarily carried out after the reaction.

Since it is extremely advantageous to achieve high level of stereocontrol in solid support synthesis, the direct roll-over method would be a suitable method to synthesize the commonly found 2-amino-2-deoxy carbohydrate moiety if we could improve the reaction efficiency. In this regard, various direct roll-over reactions were performed in solution phase to define the limits of this reaction (Scheme 2). First, the coupling reaction between iodosulfonamide **7**⁹ and lactal acceptor **6** was tried. Iodosulfonamide **7** and activated stannyl derivative of **6** were mixed in the presence of promoter silver tetrafluoroborate (AgBF_4) to yield coupled product **8**. In this particular coupling, the reaction was inefficient. Even in the presence of 10 equivalent of **6**, only 21 % yield was achieved.¹⁰ Steric congestion around the reaction site caused by the presence of neighboring fucosyl moiety was presumed to be the culprit of this lack of reactivity.

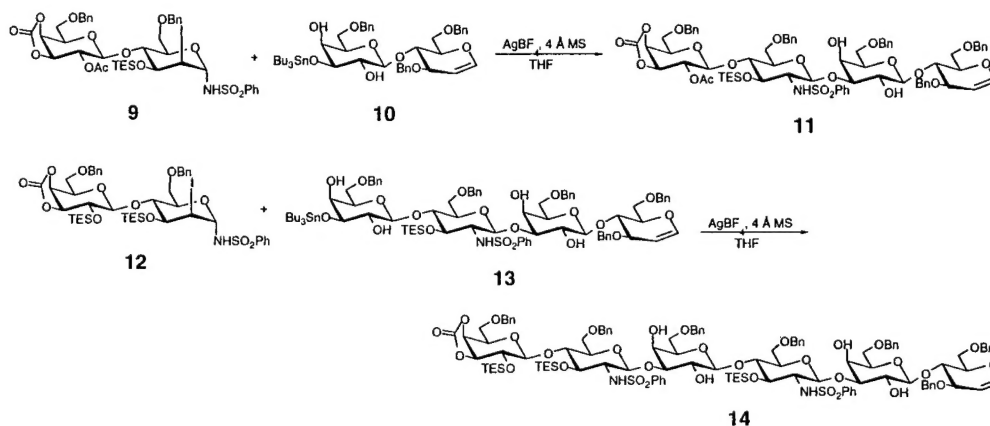
Scheme 2



Also, the reaction between **9** and **10** was carried out under similar conditions (Scheme 3). This reaction proved to be efficient. With only 4 equivalents of acceptor **10**, the coupled product

11 was obtained with a yield of 84 %. The coupling between **12** and 4 equivalents of **13** was attempted, again in presence of AgBF_4 , to yield **14** in 62 % yield. Thus, this reaction was concluded to be very sensitive to the steric environment around the reaction site, and depending on the steric nature of the donor, the direct roll-over reaction seemed to be promising even in the case of solid support synthesis. The success of the coupling reactions between **9** and **10**, and again **12** and **13** allowed a concise synthesis of nonasaccharide tumor associated antigen KH-1.¹¹

Scheme 3



Simultaneously with these model studies, significant amounts of Globo-H **1** and Globo-H allyl glycoside **2** were synthesized according to the established protocol by solution phase synthesis² and supplied to our colleagues in the immunology department.

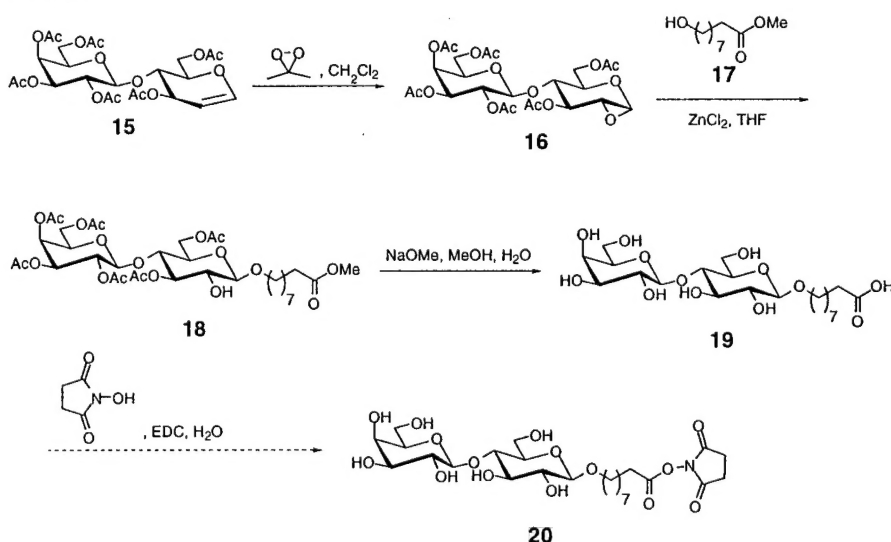
II. Conjugation of Carbohydrates to Proteins.

Another aspect of solving the Globo-H supply problem for immunocharacterization is to improve the conjugation efficiency. Because of low immunogenicity of carbohydrate antigens, they are typically conjugated to immunogenic carrier proteins.¹² In this study, keyhole limpet hemocyanin (KLH) was chosen to be a suitable protein carrier of Globo-H. Using the

conventional reductive amination method, we were able to launch preliminary biological studies of this antigen. However, the conjugation efficiency was at the low end of practicality, and other methods were sought to increase the yield of conjugation.

One of the methods devised was N-hydroxy succinimide ester method. Since this new method had to be tested on a more readily accessible substrate rather than on valuable Globo-H itself, per-acetylated lactal **15** was chosen as a model (Scheme 4). Thus, the solution of lactal derivative **15** in methylene chloride was treated with dimethyldioxirane in acetone at 0°C under N₂ atmosphere for 30 minutes to yield the lactal epoxide **16**. This epoxide **16** was subsequently treated with a solution of hydroxy ester **17** in THF in the presence of zinc (II) chloride to give lactose ester **18** in 50 % yield. Acetyl groups of **18** were removed at the same time the ester was saponified by treatment with sodium methoxide in methanolic water to yield the acid **19**. The conversion of acid **19** to N-hydroxy succinimide ester **20** was tried. However, no detectable amount of N-hydroxy succinimide ester **20** could be isolated, even though various conditions were screened. The method of generating it *in situ* in the presence of the carrier protein KLH

Scheme 4



will be tried as a possible solution to this problem. Also, other methods to activate the acid to be coupled to the protein will be tested.

Conclusion:

Two ways to improve the supply of Globo-H antigen for immunological characterization, namely the solid support synthesis of Globo-H and development of a new conjugation method, have been actively pursued while enough material for preliminary clinical study was synthesized by solution phase methodology. Both endeavors still require more refinements and further studies to achieve the ultimate goal. However, the preliminary results are promising, and the research will be continued.

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9. Made from commercially available lactose octaacetate in 7 steps.
10. Sample procedure for direct roll-over couplings: Coupling between **9** and **10**: To a solution of compound **10** (0.0983 g, 0.170 mmol) in dry benzene (90 mL) was added bistributyltin oxide (0.0500 mL, 0.0935 mmol), and the resulting solution was heated at reflux overnight with azeotropic removal of water. Thus formed tin ether was concentrated with a stream of dry N₂ and then further dried *in vacuo*. To a mixture of azeotropically dried (3 x 5 mL benzene) compound **9** (0.0405 g, 0.0425 mmol) and freshly flame dried 4 Å M.S.(0.8 g) was added a solution of the tin ether in 1.8 mL THF via cannula. The resultant suspension was cooled to -60 °C, and treated with a solution of AgBF₄ (0.0337 g, 0.170 mmol) in 0.6 mL THF via cannula. The reaction mixture was stirred for 2 days with exclusion of light while slowly allowed to warm to room temperature. The reaction mixture was diluted with EtOAc (100 mL) and filtered through a pad of silica gel. The filtrate was washed with a saturated solution of NaHCO₃ (3 x 60 mL), and brine (1 x 60 mL). The organic layer was separated,

dried (Na_2SO_4), filtered, concentrated, and submitted for chromatography (45% EtOAc in hexanes) to afford 0.0498 g (84%) tetrasaccharide **11**. Further chromatographic separation (80% EtOAc in hexanes) afforded 0.0480 g unused acceptor **10**.

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Acronyms:

Ac: acetyl.

Et : ethyl.

Bu : butyl.

$\text{I(coll)}_2\text{ClO}_4$: iodonium bis(sym-collidine) perchlorate.

LHMDS : lithium bis(trimethylsilyl)amide.

TBDPS : t-Butyldiphenylsilyl.

Bn : Benzyl

THF : tetrahydrofuran.

TES : triethylsilyl.

Me : methyl.